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One-Carbon Metabolism Related B-Vitamins Alter The Expression Of MicroRNAS And Target Genes Within The Wnt Signaling Pathway In Mouse Colonic Epithelium

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ONE-CARBON METABOLISM RELATED B-VITAMINS

ALTER

THE EXPRESSION OF MICRORNAS AND TARGET GENES

WITHIN THE *WNT* SIGNALING PATHWAY

IN MOUSE COLONIC EPITHELIUM

A Thesis Presented

by

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ABSTRACT

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It has been widely recognized that microRNAs are involved in nearly all cellular processes that have been investigated and contribute to a variety of diseases including cancer. Our prior studies demonstrated the depletion of one-carbon metabolism related B-vitamins, including folate, vitamin B2, B6 and B12, induced a genomic DNA hypomethylation and an elevation of the tumorigenic *Wnt* signaling in mouse colonic epithelium. The present study aimed to define whether microRNAs serve as mediators between these B-vitamins and the *Wnt* signaling, and thereby influence intestinal tumorigenesis. MicroRNA expression profiles were measured using miRNA microarray and real-time PCR on colonic epithelial cells from Apc^{1638N} mice fed with diets deplete or sufficient in those B-vitamins. *In silico* bioinformatic analysis were performed to predict microRNA gene targets within the *Wnt* signaling cascade. Out of 609 microRNA examined, 18 microRNAs were found to be either significantly ($p < 0.05$) or mildly ($p < 0.10$) differentially expressed in the colonic epithelium of mice fed the depleted diet when compared to the counterpart. Bioinformatic prediction of microRNA gene targets identified 40 genes within the *Wnt* pathway to have homology with microRNA seed sequences within their 3'-UTR or

protein coding sequence. Of the 6 genes tested for experimentally target validation, the expression of *Sfrp1* was shown to be significantly inhibited ($p < 0.05$) whereas β-catenin was shown to be significantly elevated ($p < 0.05$) with alterations of others in a fashion indicating the activation of *Wnt* signaling. These findings indicate that microRNAs may constitute a mechanism by which one-carbon B-vitamin depletions regulate the *Wnt* signaling pathway and thereby inform intestinal tumorigenesis.

Key words: One-carbon metabolism, microRNA, *Wnt* pathway, Colorectal cancer

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER 1

LITERATURE REVIEW

1.1 microRNA

microRNA (miRNA) are a class of short, non-coding RNA ~18-25 nucleotides in length which post-transcriptionally regulate mRNA expression in plants, animals and protozoa. Description of the *C. elegans* miRNA *lin-4* in 1993 was the first characterization of a miRNA, offering the first insight into the regulatory role of non-coding RNA (Lee 1993). Regulatory function by miRNA was not described again until 2000 with the discovery of *let-7*, also in *C. elegans* (Reinhart 2000). Both miRNA were found to repress expression of various protein coding genes through imperfect complimentary basepairing with the 3' UTR of mRNA (Reinhart 2000). These studies revealed key functions of miRNA, specifically temporal control of developmental events by miRNA through the regulation of interdependent genes (Reinhart 2000). Since these findings, identification of miRNA has increased exponentially to over 28,000 mature sequences registered in the miRBase database (www.miRBase.org, access on March 18, 2016), of which 2,588 have been identified in humans and 1,915 in mice. It is predicted that 60% of all protein coding genes in the mammalian genome harbor target site in their 3'UTR for miRNA binding (Bushati 2007) (Sayed 2011). This ubiquitous nature indicates an important role for miRNA mediated regulation of cellular processes.

1.1.1 microRNA biogenesis and function

In the nucleus RNA polymerase II generates a primary stem-loop structure (pri-miRNA) varying in size from a few hundred nucleotides to kilobases in length (Bushati 2007). Once transcribed, pri-miRNA are processed in the nucleus by a protein complex known as the microprocessor. The microprocessor consists of the RNase III, DROSHA, and doublestranded

RNA binding domain (dsRBD) protein DGCR8 dimer. DROSHA recognizes and binds the ssRNA-dsRNA junction of pri-miRNA and uses this site as a molecular ruler to identify the cleavage site ~11 base pairs from the ssRNA-dsRNA junction. DGCR8 plays three distinct roles in pri-miRNA processing: binding and stabilization of the C-terminal tail, recruitment of the pri-miRNA via the dsRBD and recognition of the apical UGU motif (Nguyen 2015). Once the pri-miRNA is bound and oriented, DROSHA cleaves the pri-miRNA at the cleavage site (Han 2006), producing a ~70 bp hairpin loop structure known as the precursor-miRNA (pre-miRNA) with a 3' 2 nucleotide overhang characteristic of RNase III mediated cleavage (Lee 2003). The 2 nucleotide overhang is subsequently recognized by the transport molecule Exportin5, (Yi 2003) which shuttles the pre-miRNA to the cytoplasm for additional processing via a Ran-GTP dependent mechanism (Bohnsack 2004)

In the cytoplasm, the RNase III Dicer anchors both the 3' and 5' end of the pre-miRNA and determines the cleavage site using the 5' counting rule. Dicer identifies the 5' phosphate cap and "measures" ~22 nucleotides from the 5' terminal end of the pre-miRNA then cleaves both strands at this site, producing a small RNA duplex (Park 2011). The RNA duplex is actively loaded onto argonaute (AGO) and unwound. Duplex unwinding then typically occurs in an ATP-independent manner. The current "rubber band" model suggests structural tension is applied in the open confirmation of AGO. Upon release tension is relieved, inducing duplex unwinding (Kawamata 2010). The strand not bound to AGO, termed the passenger strand, is then released and degraded (Matranga 2005).

The remaining strand bound to AGO, termed the guide strand, has a complimentary sequence to its target mRNA and maintains a conformation preferential for target binding (Czech 2009). The guide strand then directs the miRNA-induced silencing complex (miRISC), which is

comprised up of AGO and glycine-tryptophan repeat–containing protein (GW182), to gene targets (Bushati 2007). Upon engaging the target mRNA AGO will take an open conformation, exposing bases 2-7 of the guide miRNA, termed the seed region, to face outward and allow for efficient recognition and base pairing to the target (Gan 2015).

Depending on the degree of complementarity between the target and the guide, miRNA employ multiple mechanisms which result in the suppression of gene expression (Hutvagner 2002) (Martinez 2004). If base pairing complementarity is near perfect, hydrolysis of the target is induced by AGO, leading to mRNA degradation by cellular exonucleases (Hutvagner 2002). Hydrolysis is mediated by only one isoform, AGO2 as it is the only catalytically active AGO in humans (Cheloufi 2010). Cleavage of phosphodiester bonds occurs between the target bases paired with bases 10 and 11 of the guide miRNA (Elbashir 2001). The two product fragments exposed to degradation by the SKI complex and 5'-3' exoribonuclease 1 or 2 (XRN1, XRN2) (Chatterjee 2011).

Non-cleavage translational repression of target genes only requires complementarity with the miRNA seed region (Filipowicz 2008). This allows miRNA to target and effectively suppress as many as 100-200 targets according to computational estimates (Krek 2005) (Lim 2005). Multiple *in vitro* models of translational repression have been proposed including cotranslational protein degradation (Nottrott 2006), inhibition of translation elongation (Gu 2009), ribosome drop-off leading to premature termination of translation (Petersen 2006), inhibition of translation initiation through interference of ribosome recruitment (Mathonnet 2007), sequestering of mRNAs in processing bodies (P bodies) void of ribosomes (Ding 2007), prevention of circularization (Zekri 2009), and deadenylation followed by decapping and subsequent

degradation (Chen 2009). Despite the success of *in vitro* studies, it is unknown whether there is a primary mechanism or multiple mechanisms working in tandem *in vivo.*

1.1.2 microRNA in cancer development

miRNA activity has been observed in nearly all cellular processes including development, metabolism, cell differentiation, proliferation and apoptosis. In accordance with their ubiquitous nature, miRNA activity has been linked 163 different diseases, and play a prominent role in colorectal cancerogenesis (Jiang 2009). In cancer, miRNA may act as either oncogenes by downregulating tumor suppressor genes or conversely act as tumor suppressors by downregulating oncogenes (Luo 2011).

Differential miRNA expression in cancer cells and tissues versus adjacent normal tissue using microarrays is a method commonly used to establish cancer related miRNA profiles and has been utilized to create a miRNA expression profile in human CRC tissue (Schetter 2013). Of 164 significantly altered miRNA in CRC, 2/3 demonstrate increased expression while the remaining 1/3 demonstrate decreased expression. This variance in expression indicates a favor towards oncogenic function of miRNA (Luo 2011). Here we highlight the current literature demonstrating both tumor suppressive and oncogenic roles of miRNA in cancerogenesis with a focus on CRC when possible.

1.1.2.1 Tumor Suppressor miRNA

Increased expression of the miR-30 family has been shown to act as a tumor suppressor in humans. Tumor suppressive activity has been suggested by significant downregulation of miR-30 family miRNA, with concurrent increased expression of epithelial to mesenchymal (EMT) genes in human pancreatic islet cells (Joglekar 2009), prostate cells (Kao 2014) and hepatocytes (Budhu 2008) (Zhang 2012). A tumor suppressive role is attributed to targeting of

EMT genes such as *vimentin* and *SNAIL* which share the complementarity with the miR-30 seed sequence (Joglekar 2009). In addition, miR-30 has been shown to reduce stem-like features in tumor-initiating breast cells through targeting ubiquitin-conjugating enzyme 9 (Ubc9) and Integrin β3 (ITGB3) (Yu 2010).

Tumor suppressive activity of miR-146a has been demonstrated in multiple cancer types including oral (Scapoli 2010), prostate (Lin 2008), breast (Bhaumik 2008), pancreatic (Li 2010), gastric (Kogo 2011) and colorectal (Mao 2014) cancers. CRC tissue (Mao 2014) and stool samples both exhibit downregulation of miR-146 (Ahmed 2013), suggesting a tumor suppressive role in the disease. miR-146 likely plays a role in suppression of metastasis as it targets SMAD4, a protein which is typically lost in CRC and is associated with EMT (Zhong 2010). Further, miR-146a expression levels have been shown to be decreased in metastatic tumor tissue when compared to primary tumor tissue (Pizzini 2013).

To date, only one study has assessed the role of miR-361-5p in CRC. This initial data suggests a tumor suppressive role as expression was decreased in CRC when compared to normal tissue and overexpression inhibited invasion and metastasis (Ma 2015).Tumor suppressive action occurs through targeting of staphylococcal nuclease domain containing-1 (SND1), which has been shown to have an oncogenic effect in CRC by downregulating *APC* (Tsuchida 2011). Interplay between miR-361-5p and SND1 acts as a feedback loop, as miR-361-5p represses SND1 expression through binding of 3'-UTR while SND1 was found to bind to pre-miR-361-5p and negatively regulating its expression (Ma 2015).

The role of miR-409-3p expression in cancer appears to be tissue specific. A tumor suppressive effect on lung adenocarcinoma (Wan 2014), bladder cancer (Xu 2013b) and CRC (Bai 2015) (Liu 2015) has been demonstrated. However, miR-409-3p has an oncogenic effect in

prostate cancer (Josson 2014). Currently, only two studies have been performed in an attempt to elucidate the role of miR-409-3p in CRC (Bai 2015) (Liu 2015). Neither study has provided a clear picture as to gene targets implicated in the tumor suppressive role of miR-409-3p in CRC. The serine/threonine kinase NLK has been verified by Liu et al as a target of miR-409-3p (Liu 2015). Other studies have shown NLK is a mediator of *Wnt* signaling through phosphorylation of TCF/LEF (Ishitani 1999). Bai et al have identified Grb2-associtated binder 1 (GAB1) as a target of miR-409-3p (Bai 2015). GAB1 is a scaffolding adapter protein which mediates cell growth and differentiation and has been implicated in tumor progression in CRC (Seiden-Long 2008). Overexpression of miR-409-3p was shown to successfully downregulate GAB1 and suppress invasion both *in vitro* and *in vivo* (Bai 2015).

The major product of stem-loop mir-139 is miR-139-5p and as such there are significant insights into its function, including downregulation and a tumor suppressive role in CRC (Shen 2012). The minor product, miR-139-3p, has been studies less extensively. However, Liu et al have assessed the effect of miR-139-3p on CRC tissue from 63 patients with pathologically diagnosed primary colon cancer (Liu 2014a). miR-139-3p levels were found to be inversely associated with survival as patients with high expression of miR-139-3p had a significantly longer survival time compared with those with low expression (Liu 2014a). The results of this study are limited due to sample size and only assessed miR-139-3p as a diagnostic marker, providing no insight into its molecular role or targets (Liu 2014a). To date no gene targets for miR-139-3p have been validated in CRC tissue (Vlachos 2015).

Downregulation of miR-125a-5p has been demonstrated in breast (Guo 2009) (O'Day 2010), ovarian (Nam 2008), lung (Wang 2009b), gastric (Nishida 2011), medulloblastoma (Ferretti 2009) and colorectal cancer (Tong 2015) tissues. Recently Tong et al demonstrated

downregulation of miR-125a-5p in CRC tissues and cell lines and validated anti-apoptotic gene targets Bcl-2, BCL2L12 and MCL1 (Tong 2015). This study also showed an induction of apoptosis and inhibition of cell proliferation as a result of miR-125a-5p overexpression (Tong 2015).

1.1.2.2 Oncogenic miRNA

miR-31-5p displays complex expression patterns in human cancers, exhibiting both oncogenic and tumor suppressive roles in disease development (Xu 2013a). In melanoma, breast and prostate cancer miR-31-5p is regularly down-regulated. In contrast it is upregulated in lung, oral and colorectal cancers (Xu 2013a). Overexpression of miR-31-5p has been observed in CRC primary tumors isolated from all clinical stages of primary tumors as well as in cell lines (Bandrés 2006) (Wang 2009a) (Cekaite 2012) (Shee 2012) (Sun 2013) (Xu 2013a). High expression levels of miR-31-5p have been associated with advanced tumor stage and poor differentiation in CRC (Schee 2012). In addition, miR-31-5p expression levels have been shown to be elevated in premalignant polyps, indicating a role in early cancer development (Cekaite 2012).

Important targets for mir-31-5p in CRC development are the tumor suppressive GTPases RhoBTB1 and RASA1 (Xu 2013a) (Sun 2013) as well as TIAM1, a guanidine exchange factor of the Rac GTPase (Cottonham 2010). miR-31-5p is also associated with mutations in the proto-oncogenic gene BRAF in CRC tissue (Nosho 2014) (Ito 2014). BRAF mutations are linked to CpG island methylator phenotype (CIMP) which is defined as widespread CpG island promoter methylation, typically in DNA repair and tumor suppressor genes (Mojarad 2013) and has been correlated with increased expression of DNA methyltransferase 1 (Etoh 2004).

Oncogenic behavior of miR-27a-3p has been observed in multiple cancer types including breast (Guttilla 2009), gastric (Liu 2009) and kidney (Chow 2010). The relationship between miR-27a and CRC however has yet to be established. To date, one study has demonstrated tumor suppressive action in CRC (Bao 2014). The role of miR-27a in CRC is further complicated by the occurrence of two natural genetic variants of the miRNA (Wang 2014b). An A/G single nucleotide polymorphism (SNP) rs895819 in miR-27a appears to be associated with CRC susceptibility in ethnic Han Chinese (Cao 2014) (Wang 2014b). Wang et al was only able to show an association between the GG genotype (Wang 2014b) while Cao et al demonstrated an association between both the GG and AG genotypes and CRC (Cao 2014). Cao et al also observed increased relative expression of miR-27a in tumor tissues from those with the GG genotype and in those carrying at least one G allele when compared to tumors from AA patients, indicating an oncogenic role (Cao 2014).

miR-92a and miR-92b belong to the miR-92 family a member of the polycistronic mir-17–92 cluster (Olive 2010). The mir-17-92 cluster was the first non-coding RNA to be described as an oncogene (He 2005) and consists of four miRNA families: the miR-17, miR-18, miR-19 and miR-92 families (Olive 2010). He et al demonstrated overexpression of mir-17-92 in conjunction with overexpression of Myc, a known oncogene, increased the rate of development of malignant lymphomas when compared to overexpression of Myc alone (He 2005). However, overexpression of Myc along with individual miRNAs of the mir-17-92 cluster did not result in accelerated onset of disease (He 2005). The mir-17-92 cluster has also been implicated in hepatocellular carcinoma (Connolly 2008), medulloblastoma (Northcott 2009), leukemia (Li 2012b), lung cancer (Hayashita 2005) and CRC (Nishida 2012).

Mature sequence miR-92a has been determined to be a key oncogenic component of the mir-17-92 cluster in the development of CRC (Tsuchida 2011). In both CRC adenomas and carcinomas mir-92a is transcribed at a higher rate than any other miRNA transcribed in the mir-17-92 cluster and directly targets the pro-apoptotic tumor suppressor BCL-2-interacting mediator of cell death (BIM) (Tsuchidad 2011). Relative expression levels of miR-92a have been shown to be significantly elevated in CRC tissues when compared to normal adjacent mucosa (Zhou 2013). Elevated expression of miR-92a has also been correlated with advanced clinical stage, lymph node metastases, distant metastases, while increased expression is an independent predictor of overall survival (Zhou 2013).

To date miR-92b has been implicated in few malignancies; non-small cell lung cancer (Lei 2014), mantle cell lymphoma (Pal 2007) and Glioma (Li 2013). miR-92b levels are significantly elevated in high-grade gliomas compared with low-grade gliomas while overexpression has been shown to increase cell proliferation (Li 2013). Increased cell proliferation has been demonstrated to be regulated through modulation of the *Wnt* pathway, specifically by direct targeting and downregulation of the *Wnt* antagonist Dickkopf-3 (*DKK3*) (Li 2013).

Initially implicated in glioblastoma over a decade ago miR-21 is one of the earliest and most highly studied oncogenic miRNA (Chan 2005). Early evaluation of miRNA expression signatures in solid tumor tissues reported overexpression of miR-21 in all six tissue types considered; breast, CRC, lung, pancreas, prostate, stomach (Volinia 2006). Since these findings, overexpression of miR-21 has been reported in head and neck (Chang 2008), ovarian (Nam 2008) and esophageal cancers (Mathé 2009), leukemia (Fulci 2007), lymphoma (Lawrie 2007),

multiple myeloma (Pichiorri 2008), osteosarcoma (Ziyan 2011), and spermatocytic seminoma (Gillis 2007).

miR-21 expression levels have been established as a reliable prognostic marker (Kjaer-Frifeldt 2012) (Zhang 2013) and a significant predictor of survival (Nielsen 2011) in patients with stage II CRC. miR-21 has multiple validated tumor suppressor gene targets in CRC including programmed cell death 4 (PDCD4) (Asangani 2008), transforming growth factor beta receptor 2 (TGFβR2) (Yu 2012), Cell division cycle 25A (Cdc25A) (Wang 2009c) Integrin-β4 (ITGβ4) (Ferraro 2014) and, along with miR-31, TIAM1 (Cottonham 2010). Modulation of the *Wnt* pathway by miR-21 may play a role in CRC as Yu et al demonstrated a 60% reduction in Axin expression and increased expression of total β-catenin, c-Myc and cyclin-D1 in HCT-116 cells overexpressing miR-21 (Yu 2012).

1.2 Colorectal Cancer and the *Wnt* pathway

1.2.1 Colorectal cancer incidence and mortality

Traditionally, CRC is divided into three patterns: sporadic, familial or inherited cases. 75-80% of CRC cases are considered sporadic, due to an apparent absence of familial history, while familial cases account for 20-25%, with the remaining 5-10% made up of inherited hereditary diseases (Amersi 2005). Significant familial history is defined: “if two or more first-degree relatives have been diagnosed with colon cancer or colon polyps at any age or if a first-degree relative has been diagnosed with colon cancer or colon polyps before the age of 60” (Amersi 2005). Inherited cases of CRC are the result of either hereditary polyposis or nonpolyposis syndromes (Haggar 2009).

1.2.1.1 Incidence

Worldwide, Colorectal Cancer (CRC) is the third most common cancer in both men and women, accounting for 10% of all cancer incidences (Stewart 2014). Since the 1980s, incidence of CRC in the United States has been rapidly declining likely due to improved screening methods leading to increased detection (Jemal 2011). This has led to an average decline in incidence of 3.4% per year between 2001 and 2010 in the US (Siegel 2014). Despite the improvement of incidence rates in the United States, as well as other Western countries, areas previously thought of as low-risk for CRC are showing drastic increases in incidence rates. Spain, Singapore and Israel have shown significant increases in recent years while Japan and the Czech Republic have now surpassed the peak incidence rates of the United States (Center 2010). This increased incidence is believed to be due to the spread of Western culture, defined as a lack of physical activity, increased intake of calorie-dense foods and increased prevalence of smoking (Center 2010).

1.2.1.2 Mortality

In the United States, CRC is the third most common cancer and the third leading cause of cancer death, resulting the death of an estimated 26,270 men and 24,040 women in 2014 (Siegel 2014). However, mortality rates in the United States are decreasing at an increasing rate. Throughout the 1990s mortality decreased at an average rate of 2% per year, compared to an average of 3% between 2001 and 2010 (Howlander 2015). This decrease in mortality has been attributed to improved pattern changes in risk factors, treatment and screening (Edwards 2010). Improvements in screening have been vital to this decrease, accounting for 53% while changes in risk factor patterns and treatment account for 35% and 12%, respectively (Edwards 2010). As CRC progresses, five-year survival decreases. Diagnosis while the disease is in the localized

stage leads to a five-year survival of 90%. This rate decreases to 68% when the disease progresses to the regional stage and drastically decreases to 10% when distant metastasis occurs (Howlander 2015). This disparity in five-year survival demonstrates the importance of screening for early diagnosis, resulting in improved survival outcomes.

1.2.1.3 Colorectal cancer and *Wnt* signaling

Intestinal epithelium is the most rapidly self-renewing tissue in adult mammals with a turnover occurring every 5-7 days in the colon (Barker 2014). Regenerative capacity of the adult epithelium is regulated by *Wnt* signaling driven stem cell renewal (Barker 2014). Consequently, an important mechanistic role for *Wnt* signaling has been recognized in the development of CRC (Barker 2014). Over 90% of colon cancers possess activating mutations within the *Wnt* pathway (Fodde 2001). A variety of mutational and epigenetic silencing events within the *Wnt* pathway have been classified, most of which abrogate *APC* function (Moser 1993) (Jasperson 2014).

1.2.2 The tumorigenic *Wnt* pathway

The *Wnt* signaling pathway is an evolutionarily conserved pathway which regulates cell-cell interactions in embryonic development, adult tissue regeneration, homeostasis and stem cell maintenance (Clevers 2006). The *Wnt* signaling pathway is a group of signal transduction pathways consisting of proteins that pass signals into a cell. The *Wnt* pathway is activated by binding a *Wnt* protein ligand to a Frizzled family receptor, which passes the biological signal to the protein dishevelled inside the cell (Nusse 1992). *Wnt* signaling is partitioned into subpathways: the canonical and noncanonical pathways. The canonical *Wnt* pathway is dependent on β-catenin, leading to regulation of gene transcription, whereas the noncanonical *Wnt* pathways are not dependent on β-catenin. There are two major noncanonical *Wnt* pathways: the planar cell polarity (PCP) pathway regulates the cytoskeleton that is responsible for the shape

of the cell and the noncanonical calcium pathway regulates calcium inside the cell (Rao 2010). The canonical *Wnt* signaling has been highly researched and plays significant role in cancer development (Clevers 2006) and will thus be the primary focus of this work.

1.2.2.1 *Wnt* Ligands

Individual discoveries in different species initially characterized *Wnt* genes as the segment polarity gene *Wingless (wg)* in *Drosophila* (Nusslein-Volhard 1980) and the mouse proto-oncogene *integrated (Int-1)* (Nusse 1982). Subsequent analysis determined *Int-1* and *wg* to be homologous, forging an interrelation between oncogenic and developmental processes (Rijsewijk 1987).

Wnt genes are expressed in all metazoan species and genomic analysis has revealed 19 *Wnt* genes in human and mouse, 7 in *Drosophila*, and 5 in *C. elegans* (Nusse 2005). Human *Wnt* proteins are defined by gene sequence rather than function, sharing an average of 35% amino acid sequence identity and a conserved pattern of 23 or 24 cysteine residues (Logan 2004). All cysteine residues are believed to occupy intramolecular disulfide bridges providing the protein with a globular structure and characteristic hydrophobicity (Janda 2012).

1.2.2.2 *Wnt* Antagonists

Several secreted proteins bind *Wnt* in the extracellular matrix acting as antagonist for *Wnt* signaling (Cruciat 2012). Overall, small protein antagonists prevent ligand–receptor interactions or *Wnt* receptor maturation (Cruciat 2012). Two mechanisms for protein mediated inhibition seem to exist; binding to the Wnt co-receptor LRP6 and direct binding of, and sequestering *Wnt* proteins. Dickkopf proteins (Dkks) (Glinka 1998), Wise/SOST (Semenov 2005) and insulin-like growth-factor binding protein 4 (IGFBP-4) (Zhu 2008) are the known proteins families which act

as *Wnt* antagonists through indirect inhibition. The inhibitors Secreted Frizzled-related proteins (sFRPs) (Leyns 1997), *Wnt*-inhibitory factor 1 (WIF-1) (Hsieh 1999) and klotho (Liu 2007b) function through direct binding of *Wnt* proteins.

The Dkk family includes four proteins: Dkk1/2/3/4, with Dkk1, Dkk2 and Dkk4 playing a role in the regulation of *Wnt* signaling (Cruciat 2012). The mechanism of action for Dkk1/2 involves high affinity binding to the *Wnt* coreceptor low-density lipoprotein receptor-related protein (LRP) 5/6 resulting in the inhibition of Wnt ligand binding (Niehrs 2001). The cystine knot–containing protein Wise/SOST has been proposed to act through similar mechanism as Dkk1/2, binding to the first two YWTD–EGF repeat domains of LRP5/6, modulating Wnt signaling in a cell context-dependent manner (Semenov 2005). IGFBP-4 abrogates Wnt signaling through direct binding of the conserved carboxy-terminal thyroglobulin domain to either of the *Wnt* coreceptors LRP6 or Frz8 (Zhu 2008).

SFRP proteins represent the largest family of secreted *Wnt* inhibitors, comprising of five members: sFRP1/2/3/4/5 (Cruciat 2012). sFRP3 was first identified as Frzb (Frizzled motif associated with bone development), as it shares homology with the N-terminal cysteine rich domain of the transmembrane *Wnt* receptor Frizzled (Fzd) (Hoang 1996). Shortly after its identification, sFRP3 was found to be a an inhibitor of *Wnt* signaling (Leyns 1997). This initial work along with others demonstrated *Wnt* binding to sFRP3 resulting in sequester of *Wnt* proteins and subsequent abrogation of signaling (Leyns 1997) (Wang 1997). The conserved cysteine rich domain in the N-terminal motif of Fzd has been proposed as the *Wnt* ligand binding domain (Bhanot 1996) as it has been shown to be necessary and sufficient for sFRP-mediated inhibition of *Wnt* signaling (Lin 1997). An alternative mode of action for sFRP-mediated inhibition has also been proposed as sFRP and Fzd have been shown to dimerize via the cysteine

rich domain (Bafico 1999), a finding supported by crystal structures of the cysteine rich domains from mouse Fz8 and sFRP3 (Dann 2001).

Overall, the mechanisms by which *Wnt* signaling is regulated in the extracellular environment have yet to be fully elucidated but appear to occur through a variety of mechanisms including the sequestering of *Wnt* proteins and binding of *Wnt* receptors. Further investigation of these agonists and antagonists will provide significant insights into the role of extracellular regulation of *Wnt* signaling.

1.2.2.3 Post Transcriptional Modification

Post transcriptional modification, particularly acylation and glycosylation, has been shown to be essential for of *Wnt* protein function (Willert 2012). Glycosylation sites are variable in *Wnt* proteins in location and number (Ching 2008). Mutagenesis of glycosylated residues of Wnt3a (Komekado 2007) and Wnt5a (Kurayoshi 2007) limited their secretion, suggesting glycosylation likely plays a role in protein folding and secretion. However, this effect is not strongly observed in Wnt1 (Mason 1992). Glycosylation of *Wnt* is likely a precursor to acylation as it has been shown nonglycosylated Wnt3a is not acylated and therefore not secreted (Komekado 2007).

1.2.2.4 Canonical *Wnt* Signaling

In the absence of *Wnt* ligand binding to Fzd/LRP5/6, β-catenin is targeted for degradation (Li 2012a). In the cell, β-catenin is typically associated with the C-terminal tail of the transmembrane cell adhesion molecule E-cadherin (McCrea, 1995). Free cytoplasmic β-catenin is recognized and degraded by a muliprotein Axin destruction complex consisting of Axin, APC, GSK3β, Dvl, CK1α, βTrCP, and the proteasome (Li 2012a). Axin acts as the central scaffold of the destruction complex, directly interacting with all other core components (Liu 2002). Inside

the destruction complex, β-catenin is phosphorylated by the serine/threonine kinase casein kinase 1α (CK1α) on S45 (Liu 2002). Phosphorylation of this residue is a primer necessary for subsequent phosphorylation of T41, S37 and S33 by glycogen synthase kinase 3β (GSK3β) (Liu 2002). Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase constituent F-box protein β-TrCP (Hart 1999) leading to ubiquitination (Aberle 1997). Upon ubiquitination β-catenin is released from the Axin complex and degraded by the proteasome, allowing for reuse of the destruction complex (Li 2012a).

Activation of *Wnt* signaling through *Wnt* ligand binding of Fzd/LRP5/6 results in the stabilization of cytoplasmic β-catenin and transduction of the *Wnt* signal (Clevers 2006). Binding of the *Wnt* ligand to LRP5/6 induces phosphorylation of five PPPSPxS motif (proline, serine or threonine, x, a variable residue) repeats by GSK3β (Zeng 2005) and CK1γ (Davidson 2005). Phosphorylation of the PPPSPxS motifs signals for sequestration of Axin (He 2004) which is recruited to LRP5/6 in the presence of a *Wnt* signal (Mao 2001). Sequestration of Axin to the membrane reduces the availability of cytoplasmic destruction complexes, resulting in β-catenin accumulation (Li 2012a). Upon stabilization and cytoplasmic accumulation, β-catenin translocates to the nucleus, activating TCF/LEF transcription factors (Molenaar 1996) (Behrens et al., 1996) and downstream *Wnt* target genes such as c-Myc (He 1998), cyclin-D1 (Tetsu 1999) and c-Jun (Mann 1999)

1.2.3 Colorectal Cancer, One-Carbon Metabolism and *Wnt* signaling

1.2.3.1 One-Carbon Metabolism

One-carbon metabolism (OCM) is a set of reactions involving B vitamins riboflavin, B6, B12 and folate coenzymes (Ulrey 2005). One-carbon vitamins act as essential cofactors in nucleotide synthesis (Kim 2000) and the epigenetic process of biological methylation (Pufulete

2003). Epidemiologic observations as well as preclinical studies suggest that diminished folate status increases the risk of colorectal carcinogenesis (Giovannucci 2002) (Choi 2000). Folate plays an essential role in methylation by remethylation of homocysteine to methionine, the precursor of *S*-adenosylmethionine, the universal methyl donor for biological methylation (Selhub 2002). The reactions by which this occur are dependent on other one-carbon vitamins; riboflavin, B12 and B6 acting as cofactors (Ulrey 2005).

One-carbon vitamins also assume critical roles as cofactors in the synthesis of nucleotides: riboflavin is a precursor for the cofactor of methylenetetrahydrofolate reductase, which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (Van den Veyver 2002); vitamin B12 is a cofactor for methionine synthase, a reaction in which 5-methyltetrahydrofolate participates as a substrate in the remethylation of homocysteine to form methionine (van den Veyver 2002); and vitamin B6 is a necessary cofactor for the inter-conversion of other coenzymatic forms of folate and the metabolism of homocysteine (Davis 2004). Therefore metabolic functions of all these one-carbon vitamins are highly inter-dependent, as depletion of one may lead to biochemical phenotypes characteristic of deficiencies of the others.

1.2.3.2 One-Carbon Metabolism and *Wnt* Signaling

Aberrant DNA methylation and DNA synthesis are among the most common molecular alterations that contribute to the development of human neoplasia (Jones 2002). Specifically in CRC, de novo methylation of the *APC* promoter region also plays an important role as a ‘‘second hit’’ in silencing *APC* expression in colorectal neoplasia (Arnold 2004). Our group has previously reported *APC* expression is also impaired by a severe degree of folate depletion: after 5 weeks of depletion, strand breaks appeared in *APC* gene and these breaks were inversely

correlated to steady-state transcriptional levels of *APC* (Liu 2007a).We have further demonstrated mild depletion of one-carbon vitamins over 16 weeks increased both macroscopic tumor multiplicity and aberrant crypt foci by 50%, a 4-fold increase in *Wnt* signaling and changes in expression of *Wnt* signaling genes (Liu 2011). Thus, there is evidence that *Wnt* signaling could be altered by dietary inadequacy of one-carbon vitamins and play a role in colorectal tumorigenesis.

1.3 microRNA and the Regulation of *Wnt* Signaling

The role of miRNA in regulating *Wnt* signaling in specific cell and tissue types has yet to be firmly defined. Research has been non-contiguous and does not present a clear perspective. Anton et al have performed a systematic screen for miRNA regulating the canonical *Wnt* pathway in HEK293 cells and observed differential expression of 38 miRNA (Anton 2010). Other insights into the regulation of *Wnt* pathway genes by miRNA have been published which implicate a role in cancer (Nagel 2008) (Saydam 2009) (Gokhale 2010) (Haug 2011) (Kim 2013).

The effect of the miR-34 family expression on *Wnt* signaling has been studied in the most depth (Kim 2011) (Cha 2012) (Kim 2013). miR-34 has effectively married the tumor suppressor p53 and *Wnt* signaling pathway (Kim 2011) miR-34 is a transcriptional target of P53 and targets highly conserved sites in the UTR of *Wnt* genes;WNT3, LRP6, AXIN2, β-catenin, LEF1 (Kim 2011). This results in suppression of TCF/LEF transcriptional activity, downregulating *Wnt* signaling related gene expression (Kim 2011). This effect has been demonstrated in breast, lung and colon cancer (Kim 2011). Specifically in CRC, expression of miR-34 suppresses Axin2

through binding at multiple 5′ and 3′ UTR sites, resulting in increased nuclear GSK-3β and decreased expression of the EMT regulator Snail (Kim 2013).

Other miRNA that have shown tumor suppressive action through the regulation of *Wnt* signaling including miR-144 in bladder cancer (Guo 2013) and miR-200a in meningiomas (Saydam 2009). miR-144 has been demonstrated to have decreased expression in bladder cancer cell lines and tissues (Guo 2013). This allows for expression of the gene target enhancer of zeste homolog 2 (EZH2), inactivating *Wnt* signaling and subsequent cell proliferation (Guo 2013). The miRNA expression profile brain cancer meningiomas shows downregulation of miR-200a (Saydam 2009). miR-200a directly targets and downregulates β-catenin mRNA and protein levels and downstream downregulation of cyclin D1 (Saydam 2009).

Tumor suppressive actions of miRNA in *Wnt* signaling are not exclusive as two miRNA, miR-135a/b (Nagel 2008) and miR-92a/b (Haug 2011), have been shown to play an oncogenic role. Upregulation of miR-135a/b was found in colorectal adenomas and carcinomas resulting in downregulation of *APC* (Nagel 2008). miR-135a/b targets the 3′ UTR of *APC*, suppressing its expression and enhancing *Wnt activity* (Nagel 2008). Targeting and downregulation of *APC* by miR-135a/b was found to be successful regardless of the mutational status of *APC* (Nagel 2008). In neuroblastoma, miR-92a/b was found to downregulate the tumor suppressive *Wnt* antagonist DKK3 via binding target sites on the 3’ UTR (Haug 2011).

1.4 Nutritional regulation of microRNA in Cancer Development

Accumulating studies have reported regulation of miRNA expression by dietary factors. The focus of these studies has largely been the effect of essential nutrients and bioactive dietary compounds, such as those found in green tea, berries and turmeric in cancer development. The

results of these studies demonstrate the ability of dietary components to regulate the expression of miRNA which target epigenetic mechanisms and play a role in downstream gene regulation.

1.4.1 Essential Nutrients: Vitamin D and retinoic acid

1.4.1.1 Vitamin D

The active metabolite of vitamin D, 1α,25-Dihydroxyvitamin D3 ($1,25(OH)_2D_3$) is a major cancer chemopreventive agent which acts through the induction of cell cycle arrest (Studzinski 1985). Exogenous $1,25(OH)_2D_3$ has been studied for its effect on the mediation of miRNA expression in various cancer cell lines and has shown success in mediating cancerogenesis (Mohri 2009) (Giangreco 2013) (Wang 2009d) (Padi 2013) (Chang 2015) (Ma 2014).

Mohri et al have investigated the biological effects of $1,25(OH)_2D_3$ binding to the vitamin D receptor (VDR) in cancer cell lines as previous data had shown gradual increase in VDR mRNA and protein expression levels in parallel with ongoing dedifferentiation in the early phase of cancerogenesis, yet the VDR mRNA decreases to lower levels in late stage carcinomas (Cross 2001). These findings indicated post-transcription regulation of VDR (Mohri 2009). Indeed, this work identified a target sequence for miR-125b in the 3'-UTR of human VDR mRNA and verified targeting of this sequence using luciferase assay in ovarian granulosa-like tumor cell line KGN and breast cancer cell line MCF-7 (Mohri 2009). Treatment of MCF-7 cells with $1,25(OH)_2D_3$ resulted in significant upregulation of VDR target genes which were markedly attenuated through overexpression of miR-125b (Mohri 2009). $1,25(OH)_2D_3$ treatment demonstrated an antiproliferative effect in MCF-7 cells which was abrogated through overexpression of miR-125b (Mohri 2009). This study confirmed posttranscriptional modulation

of VDR through miRNA targeting and demonstrates a diminutive role of miR-125b on the antiproliferative effect of $1,25(OH)_2D_3$.

miR-125b, along with miR-100 expression have also been shown to be modulated by $1,25(OH)_2D_3$ treatment in pancreatic cancer cells (Giangreco 2013). Expression levels of both miR-125b and miR-100 were markedly reduced in both tumor tissues and primary prostate cancer cells when compared to benign tissues and cells, respectively (Giangreco 2013). $1,25(OH)_2D_3$ treatment mediated upregulation of miR-125b and miR-100 and was VDR dependent (Giangreco 2013). Cancer invasiveness decreased with transfection of pre-miR-125b and pre-miR-100 in the absence of $1,25(OH)_2D_3$ and was further reduced in $1,25(OH)_2D_3$ treated cells (Giangreco 2013).

In HL60 leukemia cells $1,25(OH)_2D_3$ treatment has been shown to decrease the expression of miR-181a and miR-181b in a concentration and time-dependent manner (Wang 2009d). $1,25(OH)_2D_3$ treatment of HL60 cells induced G_1 cell cycle arrest, downregulation of miR-181a and miR-181b and increased expression of tumor suppressive Cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$), a cell cycle inhibitor (Wang 2009d). Transfection of pre-miR-181a abrogated of $1,25(OH)_2D_3$ induced expression of $p27^{Kip1}$ and reduced G_1 cell cycle arrest in HL60 cells (Wang 2009d). This suggests a role for miR-181 in cell cycle regulation which may be modulated by $1,25(OH)_2D_3$.

Evaluation of CRC cells have shown induction of miR-22 by $1,25(OH)_2D_3$ treatment in a time-, dose- and VDR-dependent manner (Alvarez-Diaz 2012). Induction of miR-22 may be vital for the effectiveness of $1,25(OH)_2D_3$, as miR-22 loss-of-function by transfection of a miR-22 inhibitor (anti-miR-22) suppresses the antiproliferative effect and increased cell migration of HCT-116 and SW480-ADH cells (Alvarez-Diaz 2012). Further, transfection of anti-miR-22

resulted in increased expression of oncogenes *NELL2, OGN*, *HNRPH1, RERE* and *NFAT5* (Alvarez-Diaz 2012).

miR-627 expression has also been shown to be upregulated in $1,25(OH)_2D_3$ treated CRC cells (Padi 2013). Treatment with $1,25(OH)_2D_3$ resulted in increased cell doubling time by three hours (Padi 2013). To confirm miR-627 plays a role in $1,25(OH)_2D_3$ mediated inhibition of cell proliferation, cells were transfected with miR-627, resulting in significantly inhibited growth of HCT-116 CRC cells (Padi 2013). JMJD1A, a histone demethylase which has been shown to promote CRC growth, was predicted and confirmed as a target of miR-627 as its overexpression results in downregulation of JMJD1A (Padi 2013).

Significant alteration of miR-145 was also found in $1,25(OH)_2D_3$ treated gastric cancer cells (Chang 2012). Decreased expression of miR-145 has been characterized in gastric cancer cells when compared to normal cells (Chang 2012). miR-145 expression was shown to be induced by $1,25(OH)_2D_3$ treatment in a VDR dependent manner (Chang 2012). As with other $1,25(OH)_2D_3$ induced miRNA, overexpression of miR-145 led to induction of cell cycle arrest (Chang 2012). Two direct targets of miR-145 were identified and confirmed: E2F3 CDK6 (Chang 2012). As with other cell types, one major effect of $1,25(OH)_2D_3$ on gastric cancer cells is the modulation of cell cycle related genes through induction of miRNA.

Differential expression analysis of miRNA in bladder cancer cells treated with $1,25(OH)_2D_3$ has yielded a novel miRNA expression profile (Ma 2015). Sixteen differentially expressed miRNA were identified including miR-10a, miR-22, miR-29a, miR-30d, miR-96, miR-125b-1, miR-126, miR-130a, miR-147, miR-147b, miR-193b, miR-335, miR-421, miR-454, miR-542–5p, and miR-1237 (Ma 2015). Biological processes regulated by these miRNA were evaluated using the PANTHER pathway analysis program and found the top three

regulated biological processes to be: protein metabolism and modification (16%), protein phosphorylation (11%) and MAPKKK cascade (10%) in 253J cells, and stress response (21%), amino acid biosynthesis (15%) and sulfur metabolism (11%) in 253J-BV cells (Ma 2015). No analysis including gene target prediction or validation was performed.

ii. Retinoic Acid

The active vitamin A metabolite retinoic acid drives neurogenesis, cardiogenesis, body axis extension, development of the forelimb buds, foregut, and eye during embryonic development and is essential for vertebrate cell-cell signaling pathways that control differentiation including *Wnt*, TGF-β and hedgehog (Duester 2008). Modulation of miRNA expression by retinoic acid has been demonstrated in multiple cell types including leukemia (Garzon 2007), neural (Chen 2014) (Welch 2007) (Foley 2011) and breast cancer (Khan 2015).

Treatment of patient derived leukemia cells with all-trans-retinoic acid (ATRA), a retinoic acid derivative, showed altered expression of nine miRNA, eight of which were upregulated (Garzon 2007). Of these eight up-regulated miRNA, let-7a-3 and let-7b transcription was shown to be activated via ATRA induced NFκB binding of the let-7a-3 promoter (Garzon 2007). Further, ATRA treated cell exhibited downregulation of Bcl2 and RAS genes which was correlated to increased activation of known miRNA regulators of those proteins, let-7a and miR-15a/miR-16-1, respectively (Garzon 2007).

In ATRA treated U87 MG glioma cells, differential expression of 28 miRNA was demonstrated (Chen 2014). Of most significance was the near 10-fold upregulation of miR-302b, which was shown to target the transcriptional regulator of glioma proliferation E2F3 (Chen 2014). Increased expression of miR-302b and subsequent repression of E2F3 expression was associated with apoptosis in a dose-dependent manner (Chen 2014). miRNA mediated E2F3

modulation by ATRA treatment has also been demonstrated in neuroblastoma cell lines (Welch 2007). Typically repressed in neuroblastoma, ATRA treated cells demonstrated significant upregulation of miR-34a (Welch 2007). miR-34 was then shown to directly target E2F3, inducing the caspase-dependent apoptotic pathway (Welch 2007).

Study of neuroblastoma cell lines treated with ATRA has also yielded insight into the tumor suppressive role of miR-10a and miR-10b (Foley 2011). ATRA treatment of SK-N-BE, LAN5 and SHSY-5Y cell lines led to overexpression of miR-10a/b and induced differentiation (Foley 2011). Ectopic overexpression of miR-10a/b similarly replicated the differentiated phenotype exhibited by the ATRA treated cells (Foley 2011). Direct targeting of nuclear receptor corepressor 2 (NCOR2), a corepressor of gene transcription which is known to suppress neurite outgrowth, by miR-10a/b resulted in neural cell differentiation and downstream repression of *MYCN* expression (Foley 2011).

1.4.2 Essential nutrients: One-Carbon vitamins

The mechanistic link between cancer susceptibility, one-carbon vitamins and epigenetics has been repeatedly shown to involve modulation of miRNA expression in the development of hepatocellular carcinoma (HCC) in rat liver (Kutay 2006) (Pogribny 2008) (Tryndyak 2009) (Starlard-Davenport 2010). This connection has also been shown in head and neck squamous cell carcinoma (Marsit 2006) and most recently CRC (Beckett 2015).

Initial work by Kutay et al identified significant downregulation of miR-122a in rats fed a diet low in methionine (0.18%) and devoid of choline and folic acid when compared to controls fed the deficient diet supplemented with 0.58% L-methionine, 0.3% choline, and 2 mg/kg folic acid (Kutay 2006). Downregulation of miR-122a expression was confirmed in primary human HCC tissue samples (Kutay 2006). Follow up study identified downregulation of tumor

suppressor miRNAs miR-34a, miR-16, and miR-127 (Pogribny 2008). In rats fed a methyl deficient diet, western blot analysis revealed increased protein levels of respective gene targets miR-34a and miR-127; E2F3 and BCL6 (Pogribny 2008). Further analysis by this group reaffirmed downregulation of miR-34a and miR-127 in the developmental stages of HCC in rats fed a methyl deficient diet in addition to showing aberrant expression of miR-200b and miR-16a (Tyndyak 2008). Corresponding upregulation of gene targets E2F3, NOTCH1, Bcl-6, and Bcl-2 proteins were confirmed and related to dysregulated apoptosis as well as upregulation of ZFHX1B, a known inducer of EMT (Tyndyak 2008). miR-155 and miR-221 were found to be upregulated in liver cells of mice fed methyl deficient diets in a subsequent study, however no gene targets were verified (Starland-Davenport 2010).

In a cultured cell study of lymphoblast cell line TK-6, aberrant miRNA expression profiles in cells grown in a methyl deficient medium was found (Marsit 2006). This effect imparted by a methyl deficient diet was reversible when adding a complete medium (Marsit 2006). Of note was significant overexpression of miR-222 in methyl deficient cells (Marsit 2006). This finding confirmed *in vivo* using human peripheral blood from individuals with head and neck squamous cell carcinoma and low folate intake which showed subjects at the bottom one percentile of dietary folate intake had significantly higher expression of miR-222 when compared to subjects at the top one percentile (Marsit 2006).

To date, analysis of the effect of methyl deficient diets on miRNA in human CRC have only been evaluated in a single study. Serum miR-21, folate and B12 were evaluated in a primary case–control cohort consisting of patients with colonoscopy confirmed adenomatous colon polyps as well as in a secondary cohort taken from a large cross-sectional cohort of 649 elderly participants who gave blood (Beckett 2015). Folate and B_{12} were measured by chemiluminescent

assay while miR-21 expression was quantified using qPCR (Beckett 2015). In the primary cohort significant upregulation of plasma miR-21 was found in women with adenomatous polyps and cases had significantly higher erythrocyte bound folate than controls, but not in men (Beckett 2015). miR-21 expression varied significantly with folate levels in both females and males in both cohorts (Beckett 2015). This finding was supported using a cultured cell models showing increased expression of miR-21 in cells treated with excess folate, even when results were corrected for proliferation (Beckett 2015).

Previous work by our group has demonstrated combined mild depletion of one-carbon vitamins (Liu 2007a) (Liu 2011) affects colorectal carcinogenesis. Mice fed a one-carbon vitamin depleted diet were found to have strand breaks in the *APC* gene (Liu 2007a), increased *Wnt* signaling, increased tumor multiplicity, increased tumor incidence and increased aberrant crypt foci *in vivo* (Liu 2011). These studies effectively demonstrate a *Wnt* signaling mediated role in the development of CRC associated with dietary one-carbon vitamin intake and provide the basis for our current work.

1.4.3 Bioactive compounds

1.4.3.1 Epigallocatechin Gallate (EGCG)

The green tea polyphenol Epigallocatechin gallate (EGCG) has been shown to suppress proliferation, invasiveness and tumor growth (Fang 2015). Microarray analysis of 328 miRNA from EGCG treated HepG2 human hepatocellular carcinoma cells revealed aberrant expression compared to control cells (Tsang 2010). Downregulation of 48 miRNA including oncogenic miR-21 and upregulation of 13 miRNA including tumor suppressive miR-16 was found (Tsang 2010). Downregulation of miR-21 has also been found in EGCG treated mouse prostate tumor tissues along with upregulation of tumor suppressive miR-330 (Siddiqui 2011).

1.4.3.2 Resveratrol

Resveratrol (trans-3,4′,5-trihydroxystilbene), a dietary polyphenolic compound found in plant sources such as grapes and berries has demonstrated promise for cancer treatment and prevention in preclinical trials (Bishayee 2009). Mediation of miRNA expression by resveratrol has been shown in several cancer cell lines (Tili 2010). Resveratrol treatment of human leukemia cells resulted in upregulation of tumor suppressor miR-663, decreasing activity of target activator protein-1 (AP-1) factors JunB and JunD and lipopolysaccharide endotoxins, leading to impaired lipopolysaccharide induced upregulation of oncogenic miR-155 (Tili 2010).

Assessment of miRNA expression profiles from SW480 CRC cells treated with resveratrol showed downregulation of 26 miRNA, including CRC-associated oncogenic miR-21 and miR-92a, and upregulation of 22 miRNA, including miR-663 (Tili 2010). Resveratrol mediated suppression of oncogenic miRNA expression was correlated with increased protein levels of their gene targets including Dicer1 and tumor suppressor factors PDCD4 and PTEN (Tili 2010). Increase in miR-663 expression was associated with downregulation of key effectors of the TGF-β pathway which is involved in cell proliferation, differentiation, migration and apoptosis (Tili 2010).

1.4.3.2 Curcumin

Curcumin is the main bioactive compound in the spice turmeric and has been demonstrate to have a prolific anti-cancer effect in recent years (Anand 2008) . A number of studies show this effect may be related to altered miRNA expression induced by curcumin treatment (Sun 2008) (Yang 2010) (Zhang 2010) (Saini 2011) (Gao 2012) (Dahmke 2013) (Kronski 2014) (Guo 2015). All studies to date indicate curcumin induces upregulation of tumor suppressor miRNA leading to subsequent downregulation of oncogenic targets (Sun 2008) (Yang

2010) (Zhang 2010) (Saini 2011) (Gao 2012) (Dahmke 2013) (Kronski 2014) with some effect exhibited on the downregulation of oncogenic miRNA (Sun 2008) (Zhang 2010) (Guo 2015).

In the initial study demonstrating this effect, Sun et al showed significant upregulation of the miR-22 and downregulation of miR-199a-3p in xBC-3 human pancreatic cancer cells (Sun 2008). Upregulation of miR-22 was associated with suppressed expression of P1 transcription factor (*SP1*) a gene associated with tumor growth and metastases and estrogen receptor 1 (*ESR1*), an important breast cancer target with a role in cellular responsiveness to estrogens and antiestrogens (Sun 2008).

In breast cancer cells, curcumin related modulation of the prometatstatic and proinflammatory cytokines CXCL1 and -2 has been shown to be controlled by upregulation of miR-181b in MDA-MB-231 cell lines (Kronski 2014). Mediation of the anti-apoptotic factor Bcl-2 by curcumin induced upregulation of miR-15a and miR-16 has been shown in cell lines MCF-7, SKBR-2 and Bcap-37 when compared to controls (Yang 2010). This pair of miRNA, miR-15a and miR-16, have also been shown to be upregulated in curcumin treated K562 and HL-60 leukemia cells and regulate the expression of Wilms' tumor 1 (WT1) an oncogenic transcription factor commonly upregulated in acute human leukemia and chronic myelogeous leukemia (Gao 2012).

In addition, targeting of Bcl-2 via curcumin induced modulation of miRNA has been shown to be exhibited in murine melanoma (Dahmke 2013). A diet consisting of 4% curcumin was fed to C57BL/6 mice injected with murine B78H1 melanoma cells, leading to a 100-fold increase in expression of miR-205-5p and a significant decrease in predicted targets Bcl-2 and proliferating cell nuclear antigen (PCNA) (Dahmke 2013).

In bladder cancer, miR-203 is frequently downregulated due to *MIR203* promoter hypermethylation (Saini 2011). Saini et al demonstrated curcumin mediated restoration of miR-203 in bladder cancer cell lines, leading to inhibited proliferation, migration, invasion and induced cell-cycle arrest, and apoptosis through targeting of the oncogenes *Akt2*, *Src*, *c-jun* and *survivin* (Saini 2011). Interestingly, restoration of miR-203 function is believed to be mediated through epigenetic modulation via hypomethylation of the miR-203 promoter as curcumin inhibits DNMT1 and induces global genomic DNA hypomethylation (Saini 2013).

Downregulation of oncogenic miRNA by curcumin treatment has been demonstrated in pancreatic (Sun 2008), andenocarcinoma (Zhang 2010) and prostate cancer cells (Guo 2015). In the multidrug-resistant human lung adenocarcinoma cell line A549/DDP, curcumin treatment was shown to induce cell proliferation and induce apoptosis (Zhang 2010). Further investigation revealed this effect to be likely due to decreased expression of miR-186-3p, however no target genes associated with this effect were established (Zhang 2010).

Most recently, the effects of curcumin on miRNA expression in prostate cancer cells has been studied. Guo et al demonstrated curcumin treatment of PC3 cells led to downregulation of miR-208 in a dose-dependent manner (Guo 2015). Downregulation of miR-208 led to increased expression of its target CDKN1A, a cell cycle regulator, resulting in decreased cell proliferation (Guo 2015).

CHAPTER 2

PURPOSE OF THE STUDY

2.1 Overview

In the United States, Colorectal Cancer (CRC) is the third most common cancer and the third leading cause of cancer death, resulting in the death of an estimated 26,270 men and 24,040 women in 2014 (Siegel 2014). Convincing evidence indicates Western lifestyle and diet are critical factors for the development of CRC. Observational studies show physical inactivity, obesity, alcohol consumption and smoking coupled with low intakes of fruits, vegetables and whole grains, as well as high intake of red and processed meats are associated with increased risk of this cancer (Durko 2014). However, a significant scientific gap exists in understanding the mechanism(s) mediating this epidemiological phenomenon. Therefore, to elucidate the cellular pathways by which environmental factors, including the critical dietary factor, mediate the development of CRC is of vital importance to combat this prevalent disease.

CRC tumorigenesis is a multistep process that results from accumulations of a complex series of genetic and epigenetic abnormalities under aberrant microenvironmental influence. A vast majority (>90%) of colon cancers in humans possess over-activation of the tumorigenic *Wnt* signaling pathway and there is strong evidence that this activation is pivotal in CRC carcinogenesis (Fodde 2001). CRC is traditionally divided into sporadic (75-80%) and familial (hereditary) cases (20 ~25%) (de la Chapelle, 2004). Among familial CRC, only ~5% are due directly to inherited genetic mutations. Approximate 20% have a positive family history but cannot be categorized into hereditary CRC syndrome (Al-Sohaily 2011) (Power 2010). Familial Adenomatous Polyposis (FAP), a hereditary CRC with mutations in the *APC* gene within the *Wnt* pathway (Kinzler, 1996), only account for a small portion (1~2% of all CRCs) (Fodde

2001). Therefore, the large portion of aberrant *Wnt* signaling as described above are not due to inherited origins and might be induced by environmental factors including diet (Narayan 2003).

The *Wnt* pathway is a cell signaling pathway mediated by the highly conserved *Wnt* family of secretory glycoproteins. *Wnt* proteins signal cell proliferation (van de Wetering 2002), polarity (Wong 1993) and fate (Miller 1996) during embryogenesis as well as regulate adult tissue homeostasis (Reya 2003). In the canonical pathway, a *Wnt* ligand binds to the membrane-bound frizzled (Fzd) receptor and a co-receptor, low density lipoprotein receptor-related protein 6 (LRP6), allowing for transduction of the *Wnt* signaling pathway (Wodarz 1998). Binding of the *Wnt* ligand to Fzd and Lrp6 recruits Axin to the cell membrane, resulting in the sequester of the Axin/Apc/GSK3β destruction complex, thereby hindering β-catenin degradation and allowing its stabilization (Clevers 2006). The β-catenin protein then translocates into the nucleus where it associates with T cell factor/lymphoid enhancer (TCF/LEF) transcription factors (Molenaar 1996) (Behrens 1996) activating transcription of downstream *Wnt* target oncogenes such as *c-Myc* (He 1998), *cyclin-D1* (Tetsu 1999) and *c-Jun* (Mann 1999).

The association between folate and CRC has been well demonstrated in epidemiological and preclinical studies (Giovannucci, 2002) (Choi 2000) (Kim 2005). Folate is required as a methyl donor to homocysteine for the synthesis of methionine, which derives the universal methyl donor (*S*-adenosylmethionine) for DNA methylation (van den Veyver, 2002). Within this process, other B-vitamins, such as vitamin B2, B6 and B12, also participate in the reactions as co-factors. Deficiency in these nutrients has also been shown to result in hypomethylation in the colon (Liu 2007). It is also demonstrated that, compared to healthy colonic epithelium, CRC cells typically exhibit genomic hypomethylation, and conversely gene-specific promoter

hypermethylation, which may act in transcriptional silencing of gene expression and thereby lead to CRC (Fearon 2010).

In the recent decade, miRNAs are of particular interest in cancer research as expression modification by miRNAs has been implicated in CRC by targeting mRNAs that encoding either oncogenes or tumor suppressors (Slaby 2009). miRNAs may stimulate cancer proliferation by affecting biological reactions related the development of cancer, such as cell proliferation, evasion of growth suppression and resistance to cell death (Ross 2011). miRNAs can regulate gene expression, but by themselves can also be regulated by epigenetic modification. miRNAs can be regulated through promotor methylation as well as histone modifications (Liu 2014). CpG island promoter hypermethylation in a number of miRNAs has been demonstrated to be a hallmark of metastasis in human cancer (Lujambio 2008).

2.2 Hypothesis and Specific Aims

A prior studies from our laboratory have demonstrated the depletion of one-carbon metabolism related B-vitamins including folate, vitamin B2, B6 and B12, so-called 'one-carbon vitamins', induced a genomic DNA hypomethylation in mouse colonic epithelium, and alterations on multiple components within the *Wnt* signaling pathway were identified (Liu 2007). Extending from these observations, another study using Apc^{1638N} intestinal cancer model coupled with a "*Wnt*-reporter" mouse model (*BAT-LacZ*) further demonstrated that combined inadequacies of one-carbon vitamins amplified colonic *Wnt* signaling indicated by elevation of the externally inserted and β-catenin-dependent "*Wnt*-reporter" gene, β-galactosidase. This reporter gene provides genuine indication of the activation of *Wnt* pathway. This study also demonstrated increased intestinal tumor incidence and multiplicity consequent to the

inadequacies of these B-vitamins and the elevated *Wnt* signaling (Liu 2011). Although Strand breaks in the mutation cluster region of the *APC* gene was indicated as a possible target by one-carbon vitamin depletions, it does not exclude other potential mechanisms and pathways which may also be mediated the one-carbon metabolisms given their critical roles in both biological methylation as well as nucleotide synthesis (Liu 2007) (Liu 2011). Strikingly, recent studies indicate that miRNAs not only possess a role in the regulation of *Wnt* signaling pathway (Anton 2011), but also target the one-carbon metabolism pathway (Stone 2011). Therefore, we **hypothesize** in the present study that combined inadequacies of one-carbon vitamins mediates the *Wnt* signaling pathway via controlling the expression of miRNAs. To test this hypothesis, we utilized colonic epithelial samples from mice fed either a one-carbon vitamin deficient diet (folate, riboflavin, B6 and B12 depletion) or their replete diet, and pursue the following two specific aims:

Specific Aim #1: Using miRNA array to identify what miRNAs are altered by one-carbon vitamin depletion.

Specific Aim #2: *In silico* prediction of miRNA target genes within the *Wnt* signaling cascade, and experimental validation of the predicted *Wnt* pathway-specific genes.

2.3 Significance

In this work we define relationships between dietary intake of one-carbon vitamins, miRNAs, *Wnt* signaling and colorectal cancer. This provides a new lens to understand the parthenogenesis of the disease in relation to dietary B-vitamin status. We discover a miRNA expression profile for colorectal epithelial tissue with one-carbon vitamin depletion. This research is the first to elucidate the effect of one-carbon vitamin deficiencies on miRNA

expression and presents the identification of their *Wnt* signaling-specific gene targets in colorectal epithelium. An understanding of the molecular and biochemical responses produced by one-carbon vitamin should be beneficiary to the public health via designing appropriate dietary strategies.

CHAPTER 3

EXPERIMENTAL DESIGN AND METHODS

Colonic samples used in this project were collected from an animal study performed in Tufts University. The protocol was approved by the Institutional Animal Care and Use Committee at the Jean Mayer U.S. Department of Agriculture (USDA) Human Nutrition Research Center on Aging at Tufts University. Brief descriptions for the animals, diets, as well as sample collections were shown below, more details regarding the experimental design can be found in our previous publication (Liu 2011).

3.1 Animals and Sample Preparation

3.1.1 Animals and Diets

The tumorigenic model, Apc^{1638N}, was intentionally selected to investigate the modulation of intestinal tumorigenesis. This model possesses a mild tumorigenic phenotype (Pretlow 2003), which is particularly useful for this type of experiment, since the very aggressive tumorigenic phenotypes present in many of the more commonly used models, such as the *Apc*^{min} mouse, readily overwhelm the modest effects produced by nutritional interventions. Both strains, which are on the C57BL/6 background, were bred and cross-bred in our center. These animals were pair-fed one of two experimental diets that contained different levels of folate and vitamin B2, B6, and B12 (*Table 1*) for 16 wk beginning at 8 wk of age. The amino acid-defined diet was originally designed to precisely govern folate status (Clifford 1989) and has been used extensively in our previous studies (Song 2000). It is noteworthy that diets intended to induce very mild degrees of dietary vitamin depletion were used in this study; this issue is discussed in greater depth in our previous publication (Liu 2011).

3.1.2 Colonocyte Isolation and Sample Preparation

After consuming the experimental diets for 16 weeks (from the age of 8 weeks to 24 weeks), all mice were anesthetized and then exsanguinated by cardiac puncture. The method for colonocyte isolation was previously described (Bjerknes 1981) and with slight modification. After washing with ice-cold PBS, the colon was inverted and incubated in 30 mM EDTA at 4°C for 30 mins. The colonocyte was then scraped off gently with forceps. The muscular and connecting tissue were discarded and the isolated colonocytes were washed with PBS. After centrifugation, the isolated colonocyte pellets collected into 1.5 ml Eppendorf tube and frozen in -80°C for the following DNA or RNA extraction and molecular assays.

Table 1 Concentrations of one-carbon vitamins in the experimental diet.

Treatment	Riboflavin (mg/kg)	Pyridoxine HCl (mg/kg)	B_{12} (μg/kg)	Folic acid (mg/kg)
Folate sufficiency (control)	6.0	7.0	5.0	2.0
Multiple vitamin depletion (MVD)	0.5	0.5	0.0	0.0

3.2 The measurement of microRNA profile

3.2.1 Affymetrix microRNA array

miRNA expression profile analysis were performed on isolated colonic epithelial cells from 6 Apc^{1638N} mice fed control or MVD diets using the Affymetrix GeneChip miRNA Array v. 1.0. The GeneChip miRNA Array v. 1.0 covers 71 organisms including mouse, rat, human, monkey, canine and others. A total of 609 mouse miRNAs were included in this Genechip. Total RNA and miRNA extraction from tissue samples were performed using using TRIzol® (Invitrogen, ThermoFisher Scientific). miRNA were labeled using the FlashTag Biotin HSR labeling kit according to manufacturer's protocol (Affymetrix, CA). First, Poly (A) tailing is performed at 37°C for 15 minutes, subsequently followed by ligation of the FlashTag biotin

signaling molecule, performed at room temperature for 30 minutes, before the addition of the Stop Solution. The labeled RNA samples were then added to a hybridization mixture and hybridized on the microarray overnight. Following hybridization, the arrays were washed and stained using the Affymetrix Fluidics Station 450 and scanned with the Affymetrix GeneChip Scanner 3000 7G using the Command Console software. Affymetrix miRNA QCTool were used for quality control, with following-up statistical analysis using SAS v9.4 as described in the statistical section.

3.2.2 Validation of microRNA array by real-time PCR.

To validate the results of Affymetrix miRNA array, we selected two miRNAs (miR21, and miR122) whose expressions were identified by the miRNA microarray to be significantly changed between the control and one-carbon vitamin depletion group. We also selected 3 miRNAs within the miR-29 family (miR-29a, miR-29b and miR-29c) whose expression were detected in the Affymetrix miRNA array, but the differences between the one-carbon vitamin depletion group and replete control group did not reach a statistically significant degree. These three miRNAs were associated with colorectal cancer (Kapinas 2010). Moreover, the family of miR-34, consisting of miR-34a, b and c, is known to regulate the processes of growth, apoptosis and metastasis (Roy 2012). However, the signals for this miR-34 family were below the detectable level in the Affymetrix microarray, we examined their expressions by real-time PCR. For the examination of these miRNAs, the production of cDNA by reverse transcription were performed using NCode^{TM} miRNA First-Strand cDNA Synthesis kit (Invitrogen, ThermoFisher Scientific). All the miRNAs in the RNA sample (2 ug) were polyadenlyated using poly A polymerase and ATP. Following polyadenylation, SuperScript III reverse transferase and a specially-designed universal RT primer were used to synthesize cDNA from the tailed miRNA

population. Real-time PCR analysis was performed on ABI prism 7300 (Applied Biosystems) using the SYBR Green master mix (Applied Biosystems, ThermoFisher Scientific). miRNA detection was performed using SYBR Green. Data was normalized using the average Ct value of two mouse housekeeper genes, sno-251 and sno-55 for miR-21, 122, 29a, 29b and 29c, whereas the Ct value of 5s rRNA were used as control for the normalization of miR34a, 34b and 34c. The selections of housekeeping small RNAs as controls for the normalization did not make significant differences. The miRNA and forward primer sequences were shown on *Supplementary Table 1*.

3.3 Prediction of microRNA target genes within the *Wnt* pathway

3.3.1 The prediction of miRNA targets by DIANA mirPath

Gene target predictions for 18 miRNAs with either significant ($p<0.05$) or mildly altered expression ($p<0.10$) identified by Affymetrix array or Real-Time PCR was performed using the DNA Intelligent Analysis (DIANA) miRNA pathway analysis webserver DIANA-miRPath v2.0. This target prediction tool provides the highest sensitivity and specificity when compared to other available miRNA target prediction tools, although this produces fewer results (Reczko 2012a). *In silico* miRNA target prediction is performed using the latest version of DIANA-microT algorithm, DIANA-microT-CDS, an algorithm which computes a miRNA–gene interaction score for predicted targets and/or experimentally verified targets derived from DIANA-TarBase v6.0. DIANA-miRPath v2.0 allows for hierarchal clustering based on interaction levels as well as pathway enrichment utilizing Kyoto Encyclopedia of Genes and Genomes (KEGG) v58.1 for *Mus musculus* (Vlachos 2012).

DIANA-microT-CDS predicted targets were utilized as few targets have been verified and thus using DIANA-TarBase v6.0 yielded few results. DIANA-microT-CDS has been

updated to utilize miRBase version 18 and Ensembl version 69 target sequences from both 3′-UTR and protein coding sequence (CDS) transcripts while other miRNA-gene interaction algorithms use 3′-UTR target sites alone (Reczko 2012b). This allows for increased sensitivity as miRNA favor target sites in the CDS in genes with short 3′-UTRs (Reczko 2012b). DIANA-microT-CDS has been demonstrated to have the highest sensitivity at any level of specificity when compared with other state-of-the-art miRNA-gene interaction algorithms when tested against pulsed stable isotope labeling by amino acids in cell culture (pSILAC) proteomics data sets (Reczko 2012a). An interaction score cut-off of 0.8 was chosen as it provides a manageable number of predicted targets, on average 350 targets per miRNA, as well as appropriate precision and sensitivity.

Enrichment analysis of miRNA gene targets within the *Wnt* pathway was performed under the selection of KEGG pathway mmu04310. This enrichment utilizes the one-tailed Fisher's exact test algorithm. This algorithm was chosen as it is used to detect enriched pathways with targets of specific miRNAs, exempting depleted pathways, is appropriate for small number statistics, and provides exact probabilities.

3.3.2 Validation of predicted miRNA targeted genes within the *Wnt* pathway

Validations of 6 predicted target genes within *Wnt* pathway selected from the *Wnt* signaling cascade: *Wnt*s and *Wnt* antagonists (*Wnt11*, *Sfrp1*), Signaling transduction genes (*Lrp1*, *APC*, *β-catenin*) and downstream genes (*Axin 2*), were performed using Real-Time PCR. Total RNA was extracted from frozen tissue using Trizol (Invitrogen, ThermoFisher Scientific) and purified using the RNeasy Mini Cleanup kit (Qiagen, Valencia, CA, USA). cDNA was then synthesized with SuperScript III (Invitrogen, Carlsbad, CA, USA). Real-time PCR was

performed on the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). Primer sequences were listed in supplementary Data (*Table S2*).

3.4 Interaction of microRNA and *Wnt* pathway

Gene target prediction data was visualized using Cytoscape v3.3, an open source bioinformatic software platform for visualizing molecular interaction networks and biological pathways and integrating these networks with annotations, gene expression profiles and other state data. The *Wnt* KEGG pathway for *Mus musculus* was downloaded from the KEGG pathway database (http://www.kegg.jp/kegg/pathway.html) and imported into Cytoscape using the KEGGscape application. Predicted miRNA-gene target interactions were then uploaded using data generated with DIANA-miRPath v2.0.

3.5 Statistical analysis

Data are expressed as means ± SEM. Data analysis was performed using SAS (Version 9.4, SAS Institute, Cary, NC). Comparisons between groups were made with a T-test. Significance was accepted when $p < 0.05$, and a false discovery rate cutoff of $q < 0.2$ was used when multiple comparisons were conducted. Heatmap was created based on transformed z-score values using R program.

For the miRNA and *Wnt* pathway related expression data analysis, the expression of each gene was normalized to the housekeeping genes, small RNAs (SnoRNA or 5s rRNA), or GAPDH ($Ct_{\text{target gene}}$-$Ct_{\text{housekeeping}}$). Statistical analyses were performed based on ΔCt. The relative gene expression in the multiple one-carbon vitamin deplete group comparing to the replete control group were reported as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct=\Delta Ct_{MVD}-\Delta Ct_{Control}$.

CHAPTER 4

RESULTS

4.1 Tumor incidence and multiplicity increased in mice fed a multiple one-carbon vitamin deficient diet

The number of tumors in the small intestine and colon, which were assessed in a blinded fashion, was significantly different between the control group and the vitamin deplete group (*Table 2*). In the control group, tumors were observed in 5 out of 10 mice, whereas 10 out of 11 mice possessed tumors in the deplete group ($p = 0.064$). Tumor multiplicity, which indicates the number of tumors per mouse, was significantly greater ($p = 0.028$) in the deplete group than in the controls. To limit the cost, only 8 animals per group were selected for the follow-up miRNA assays and the tumor incidence and multiplicity of these animals were shown in the parentheses in *Table 2*.

Table 2 Influences on tumorigenesis. The effect of one-carbon vitamin deficiencies on tumor incidence (percentage of mice bearing tumors) and tumor multiplicity (average number of tumors per mouse in each group) in Apc^{1638N} mice.

Diet	Tumor Incidence	Tumor Multiplicity
CTRL	5/10 (3/8)*	0.500 (0.375)
MVD	10/11 (8/8)	1.364 (1.625)
***p*_value**	0.064	0.028

* Numbers in the parentheses represent tumor incidence and tumor multiplicity of the mice in each group selected for the miRNA assays. CTRL: Control; MVD: Multiple Vitamin Depletion.

4.2 One-carbon vitamin deficiencies altered the microRNA expression profile in the colonic epithelium

4.2.1 miRNAs identified from Affymetrix miRNA microarray

The miRNA expression profiles were measured using Affymetrix miRNA microarrays on colonic epithelial cells from Apc^{1638N} mice fed with one-carbon vitamin deficiencies (folate, vitamin B2, B6 and B12) or diets sufficient in those B-vitamins. Six miRNAs (miR-30e-3p, miR-122, miR-711, miR-541, miR-92b and miR-21) in a total of 609 examined to be significantly altered in MVD mice ($p<0.05$) with additional 9 microRNAs having a mild degree of alterations ($p<0.10$). The fold changes of these miRNAs were shown in Supplementary Data (*Table S3*)

Following array analysis, samples were clustered according to their miRNA profile using the R software hierarchical clustering algorithm. Hierarchical clustering analysis revealed two distinct clusters, composing of those that are upregulated and those that are downregulated in MVD deficient mice when compared to controls. Upregulation of 7 miRNAs (miR-21, miR-711, miR-541, miR-30e-3p, miR-409-3p, miR-146a, miR-27a) was observed in MVD mice when compared to controls. In comparison we observed downregulation of 8 miRNA (miR-361, miR-690, miR-31, miR-92a, miR-92b, miR-139a-3p, miR-125a-5p, miR-122) in MVD mice compared to controls (*Figure 1*).

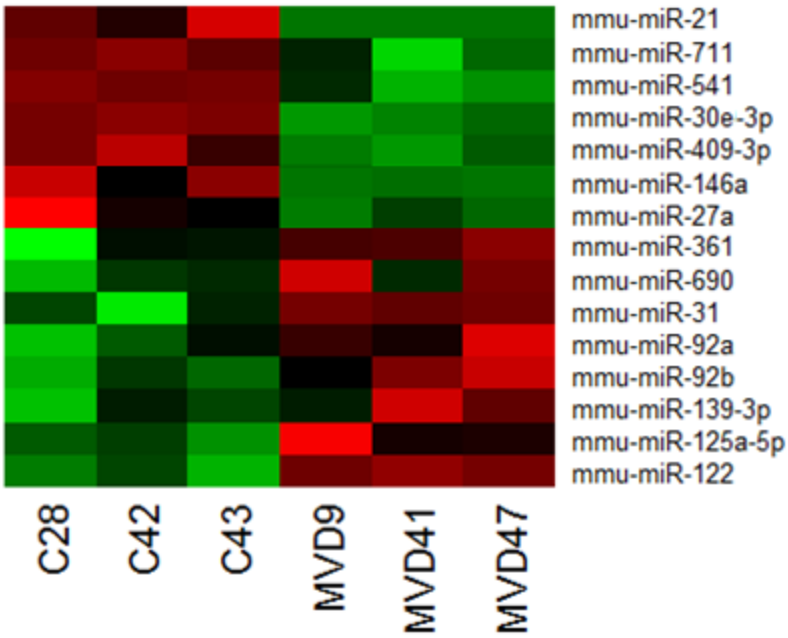

Figure 1 Differential expression of miRNA. Changes in expression levels in miRNAs were induced by one-carbon vitamin deficiencies in Apc^{1638N} mice when compared to controls. 15 miRNA either significantly ($p<0.05$) or mildly altered ($p<0.10$) through one-carbon vitamin deficiencies were identified: 7 miRNAs (miR-21, miR-711, miR-541, miR-30e-3p, miR-409-3p, miR-146a, miR-27a) were upregulated in MVD mice when compared to controls. 8 miRNA were down regulated (miR-361, miR-690, miR-31, miR-92a, miR-92b, miR-139a-3p, miR-125a-5p, miR-122) in MVD mice compared to controls. Heatmap was generated using Euclidean measure to obtain distance matrix and complete agglomeration method for hierarchical clustering. Green indicates upregulated expression, black indicates 0, and red indicates downregulated expression.

4.2.2Validation of microRNA array by real-time PCR

For the two miRNAs (miR-122, and miR-21) whose expressions were identified by the Affymetrix miRNA microarray to be significantly changed between the control and one-carbon vitamin depletion group, our real-time PCR validation recapitulates the microarray data, and significant differences were identified (*Figure 2*). For those 3 miRNAs within the miR-29 family (miR-29a, miR-29b and miR-29c) whose expression were detected in the Affymetrix miRNA array, but the differences between the one-carbon vitamin depletion group and replete control group, our real-time PCR results showed a same pattern as the Affymetrix miRNA microarray data: the expression of all 3 miRNAs increased by the depletion of one-carbon vitamins, but the real-time PCR detected a statistical significant increase for miR-29c ($p=0.045$), and a mild degree ($p=0.060$) of increase for miR-29b (*Figure 3*). For the 3 miRNAs within the family of miR-34 (miR-34a, miR-34b and miR-34c), the signals of which were below the detectable level in the Affymetrix microarray, our real-time PCR results showed a significant decrease ($p < 0.05$) of the miR-34c expression in the MVD group (*Figure 4*).

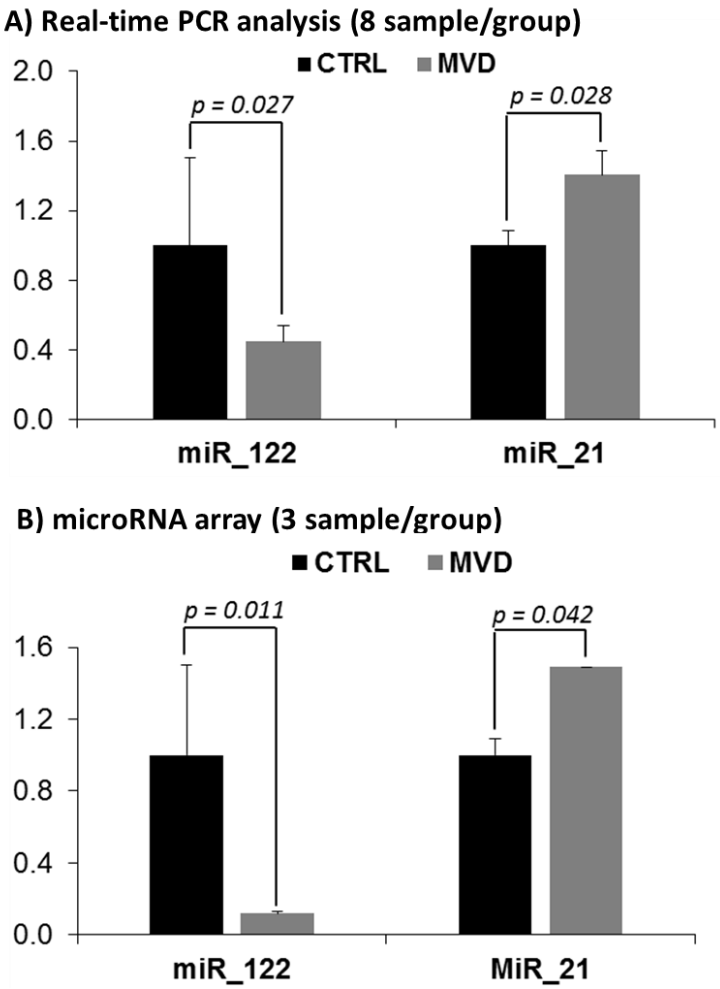

Figure 2. Relative expression of miR-122 and miR-21 in colonic samples from mice with and without multiple one-carbon deficiencies from real-time PCR and Affymetrix microarray Analysis. A) Real-time PCR results. One-carbon vitamin deficiencies resulted in a 60% decrease in expression of miR-122 (p=0.027) and a 40% increase in miR-21 expression (p=0.028). B) Affymetrix miRNA microarray results. One-carbon vitamin deficiencies decreased expression of miR-122 by 90% (p=0.011) and increased expression of miR-21 by 50% (p=0.042).

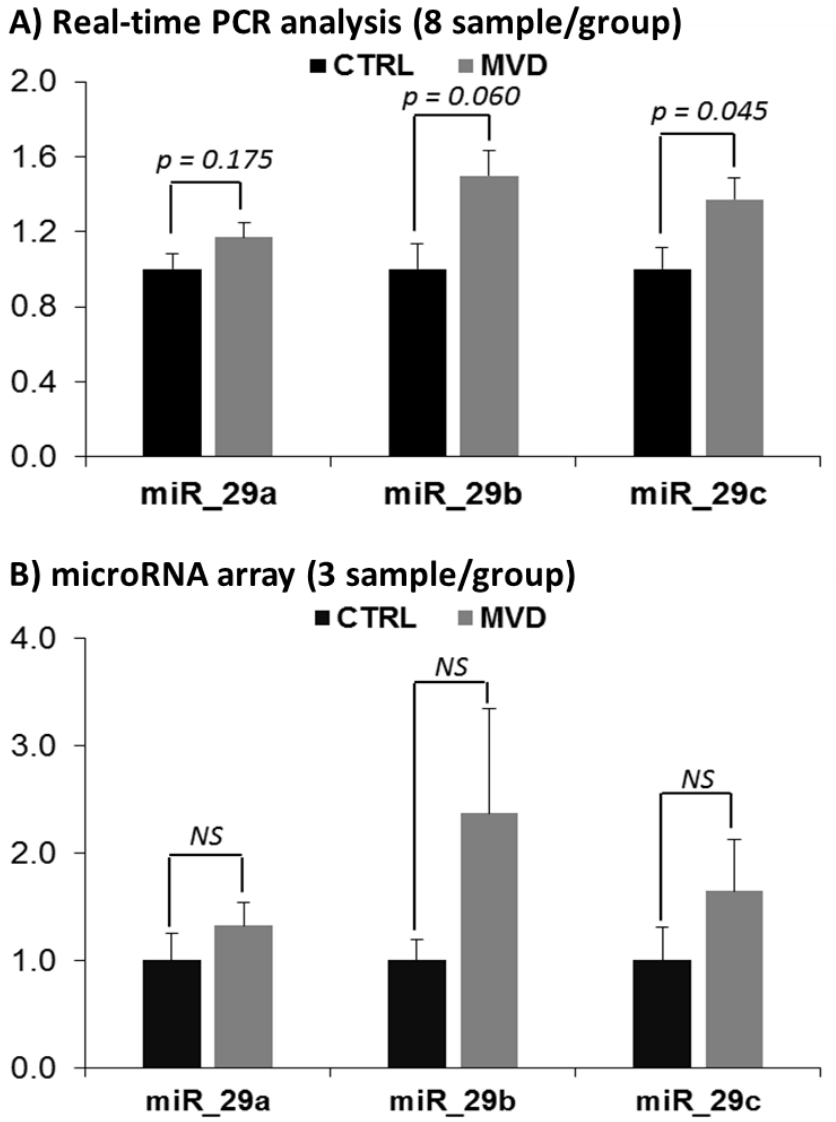

Figure 3. Relative expression of the miR-29 family in colonic samples from mice with and without multiple one-carbon deficiencies from real-time PCR and Affymetrix microarray analysis. A) Real-time PCR results. One-carbon vitamin deficiencies resulted in increased expression of miR-29a by 20% (not significant), miR-29b by 50% (not significant) and miR-34c by 50% (p=0.045) B) Affymetrix miRNA microarray results. Increased expression was observed in all miR-29 family members, however this increase was not significant.

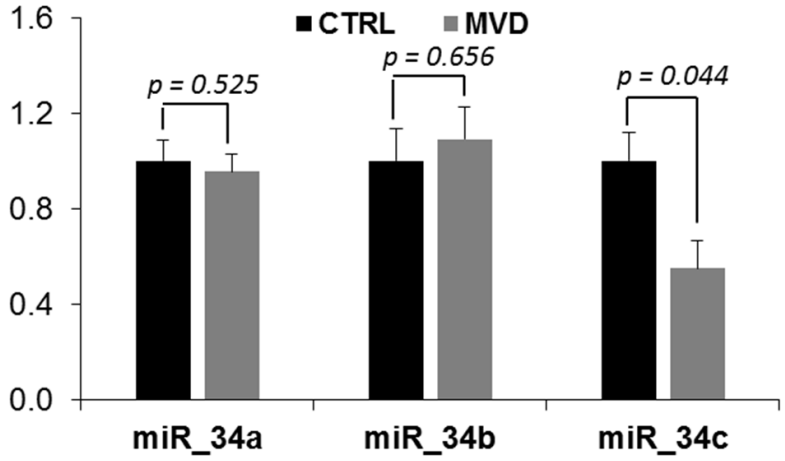

Figure 4. Relative expression of the miR-34 family in colonic samples from mice with and without multiple one-carbon vitamin deficiencies from real-time PCR analysis. One-carbon vitamin deficiencies decreased expression of miR-34c by 50% (p=0.044).

4.3 The prediction of microRNA targets within the *Wnt* pathway

4.3.1 Predicted miRNA target genes.

A total of 18 miRNAs, including those 15 miRNA which were shown to be either significantly ($p < 0.05$) or mildly altered ($p< 0.10$) in the Affymetrix miRNA microarray data, plus miR-29b, miR-29c and miR-34c identified to be significantly ($p < 0.05$) or mildly ($p< 0.10$) by real-time PCR, were assessed for gene targets within the *Wnt* pathway using the DIANA mirPath tool. A total 2,074 total predicted gene targets across 85 different pathways, of which 15 of 18 miRNA tested were shown to have a total of 40 gene targets within the *Wnt* pathway ($p<0.05$). Of the 40 predicted genes, 26 are associated with canonical β-catenin pathway, 12 are associated with the PCP pathway and 14 are associated with the Ca2+ pathway (*Figure 5*). A detailed list of those miRNAs and their target genes within the *Wnt* pathway is shown in the Supplementary data (Table S4).

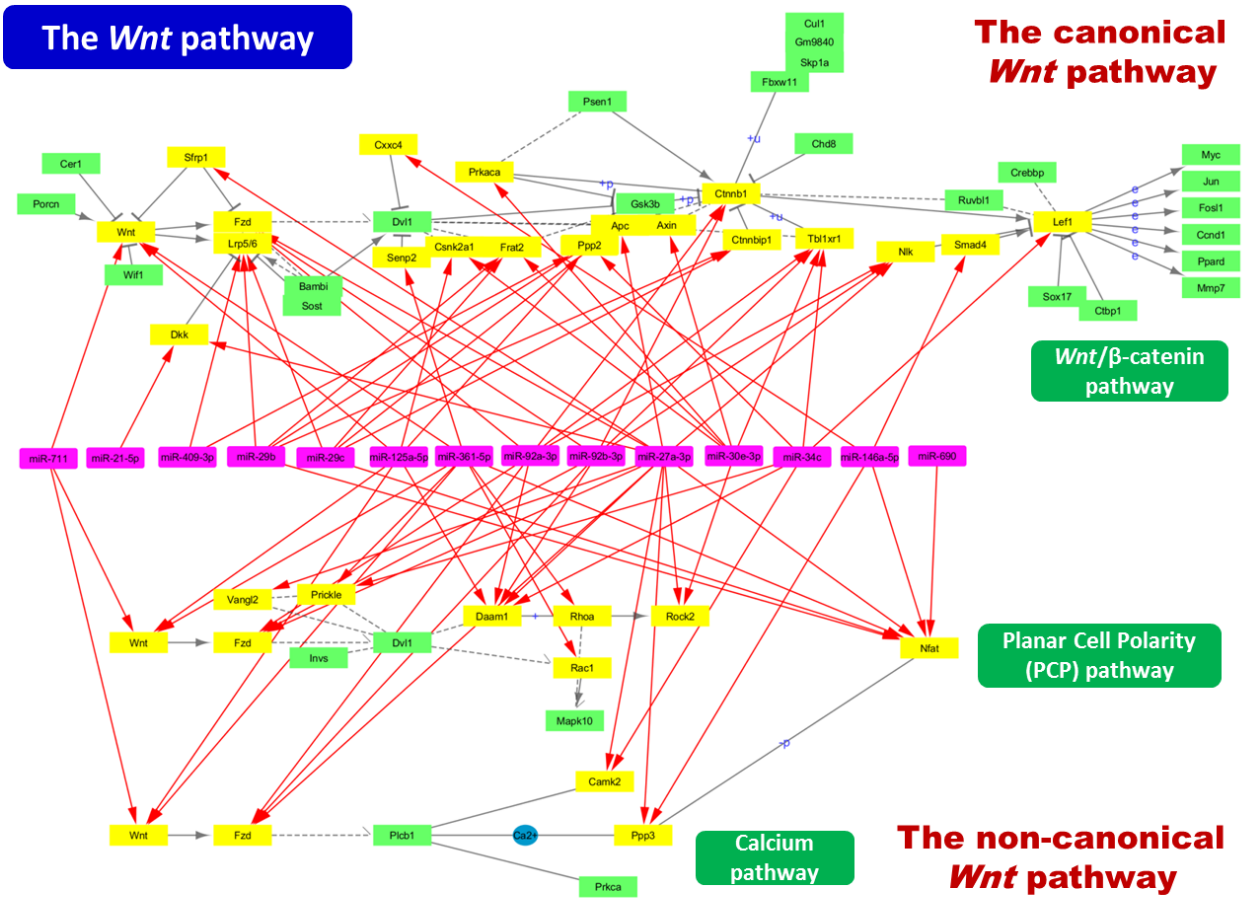

Figure 5. microRNA gene targets within the *Wnt* signaling pathway. 18 significantly altered (P<0.05) or mildly altered (P<0.10) miRNA in Apc^{1638N} mice fed a one-carbon vitamin deficient diet are predicted to have a total of 40 gene targets within the *Wnt* pathway (P<0.05). Targets were predicted using gene target sequences from both 3′-UTR and protein coding sequence *in silico* using DIANA microT-CDS. KEGG pathway enrichment was performed using a one-tailed Fisher's exact test and corrected for false discovery rate. No *Wnt* signaling associated gene targets were predicted for miR-122, miR-139 or miR-541. Three previously validated *Wnt* signaling associated gene targets for miR-122 were found when using DIANA Tarbase: Camk2b, Ccnd1 and Smad4. 14 miRNA are shown in fuchsia, targeting is shown by red arrows, predicted gene targets are shown in yellow.

4.3.2 Validation of the microRNA predicted genes within the *Wnt* pathway

To experimentally validate the *in silico* predicted miRNA target genes, we measured 6 target genes selected from the *Wnt* signaling cascade: *Wnt*s and *Wnt* antagonists (*Wnt11, Sfrp1*), Signaling transduction genes (*Lrp1, APC, β-catenin*) and downstream genes (*Axin2*) with real-

time PCR using the ViAA™ 7 PCR system (Applied Biosystems, Carlsbad, CA). Our results showed that one-carbon vitamin deficiencies elevated the expressions of β-catenin ($p < 0.05$) and increased the expression of the *Wnt* pathway downstream gene, *Axin2*, to a less degree ($p = 0.054$). We also observed, the expression of *SFRP1* was inhibited ($p < 0.05$). The overall expressions of the *Wnt* pathway-specific target genes examined altered in a fashion indicating the activation of *Wnt* signaling (*Figure 6*).

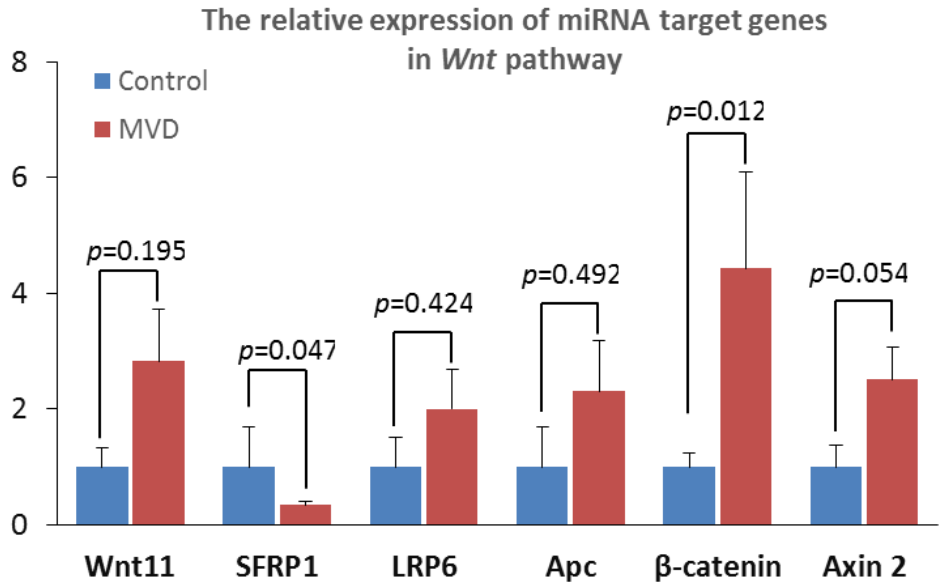

Figure 6. Relative Expression of miRNA target genes within the *Wnt* signaling cascade associated in colonic samples from mice with and without multiple one-carbon vitamin deficiencies determined by real-time PCR. One-carbon deficiencies increased expression of *β-catenin* and *Axin2* 4.4-folds ($p < 0.05$) and 2.5 folds ($p=0.054$) respectively, whereas decreased the expression of *SFRP1* by 2.9 folds ($p < 0.05$) (Control n=8 mice; MVD n=8 mice).

CHAPTER 5

DISCUSSION

Over the past decade, the functions of miRNAs have been intensively investigated, and it is well-accepted now that miRNA regulate a variety of biological processes including development, metabolism, cell differentiation, proliferation and apoptosis, and thereby contribute to nearly all types of diseases including cancer (Jiang 2009). Our prior studies demonstrated the depletion of one-carbon metabolism related B-vitamins, including folate, riboflavin, vitamin B6 and B12, induced a genomic DNA hypomethylation and an elevation of the tumorigenic *Wnt* signaling in mouse colonic epithelium. The present study showed that the combined inadequacies of those one-carbon vitamins alters the expressions of a number of miRNAs, and an *in silico* bioinformatic analysis indicated that multiple components within the *Wnt* signaling cascade are targeted by those miRNAs identified. These findings indicate that miRNAs may constitute a mechanism by which one-carbon B-vitamin depletions regulate the *Wnt* signaling pathway and thereby inform intestinal tumorigenesis.

In this study, using the miRNA microarray (GeneChip miRNA Array v. 1.0, Affymetrix) which contained 609 mouse miRNAs, 208 miRNAs were detected in at least 4 samples out of the total of 6 samples examined. Among these miRNAs, we identified 6 miRNAs (miR-30e-3p, miR-122, miR-711, miR-541, miR-92b and miR-21) to be significantly altered in the B-vitamin deplete group ($p < 0.05$) with additional 9 miRNAs having a mild degree of alterations ($p < 0.10$), indicating that only ~3% miRNAs were significantly altered by one-carbon vitamin depletions with additional ~4% miRNAs altered in a mild degree. Though the total percentage of miRNAs identified to be altered is not high, the data is consistent with prior studies which also showed a methyl deficient diet altered 1~7% miRNAs in liver (Kutay 2006) (Tryndyak 2009) (Dolganiuc 2009).

To validate the miRNA microarray data, we performed real-time PCR individually for 2 miRNAs (miR-21 and miR-122) which were identified to be significantly altered by the microarray, 3 miRNAs (miR-29a, miR-29b, and miR-29c) whose signals were detectable in the microarray, but not significantly different between the B-vitamin deplete and replete groups, and 3 miRNAs (miR-34a, miR-34b, and miR-34c) whose signals were not detectable in the array. Consistent with the microarray data, significant differences were detected for miR-21 and miR-122 by real-time PCR. For the miR-29 family, our real-time PCR measurement showed a similar pattern with the microarray data, but the differences between the dietary treatments reached to be significant or mildly significant for miR-29c (p = 0.045) and miR-29b (p = 0.060). For miR-34 family, the real-time PCR identified the expression of miR-34c was significantly downregulated by one-carbon vitamin depletion (p = 0.044). We performed real-time PCR on 8 samples per group rather than 3 samples per group for the microarrays, and the sample size increased the power to statistically identify the differences. Nevertheless, new miRNAs continue to be defined and up to date there are 1915 mature miRNAs are discovered (www.miRBase.org, access on March 18, 2016). It should be expected that there are more miRNAs whose expressions may be regulated by one-carbon vitamins.

Here we present a novel miRNA expression profile for murine colonic epithelial tissue in animals fed a one-carbon vitamin deficient diet. Interestingly we find significant upregulation of miR-21 in both microarray and PCR analysis. When compared to other miRNA, miR-21 has been studied with notable depth, particularly as an oncogene in CRC. miR-21 has been established as a reliable prognostic marker (Kjaer-Frifeldt 2012) (Zhang 2013), is a significant predictor of survival (Nielsen 2011) and has multiple validated tumor suppressor gene targets in CRC (Allgayer 2009) (Wang 2009e) (Cottonham 2010) (Chang 2011) (Yu 2011) (Ferraro 2014).

Wnt associated gene target prediction for miR-21 conferred predicted homology for a single gene, Dkk2. *In vitro* expression of Dkk2 has previously been shown to be significantly downregulated by overexpression miR-21 human tongue cancer cell line SCC25 (Kawakita 2014).

Another miRNA identified by both microarray and real-time PCR is miR-122. However, our *in silico* prediction did not find any targets within the *Wnt* pathway for miR-122. Target prediction using DIANA microT-CDS which utilizes 3'-UTR and protein coding sequence data from miRBase version 18 and Ensembl version 69 to generate a miRNA-gene target interaction score yielded zero results. This was particularly surprising as current research suggests miR-122 acts as a tumor suppressor through direct targeting of the *Wnt* ligand (Xu 2012) (Wang 2014a). In both glioma (Wang 2014a) and hepatocellular carcinoma miR-122 (Xu 2012) has been shown to directly target *Wnt1* with an identified target binding site in the 3'-UTR. This result is not limited to DIANA microT-CDS; other well established tools such as Targetscan v7.0 (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna) did not predict *Wnt1* as a top 1000 targets either (data not shown). However, target prediction using DIANA Tarbase v6.0, which utilizes verified gene targets, yielded three genes: *Camk2b*, *Ccnd1* and *Smad4* but did not include *Wnt1* (data not shown). These findings suggest there are limitations to the use of *in silico* miRNA gene target prediction tools, even when gene targets have been previously verified.

With the limitations of *in silico* miRNA gene target prediction in mind, we experimentally validated 6 genes selected from sections of the *Wnt* signaling cascade: *Wnt*s/*Wnt*s antagonists, signaling transduction genes, and downstream genes. Our results showed that the expression of *Sfrp1* was inhibited ($p < 0.05$). *Sfrp1*, a *Wnt* signaling antagonist, has been

previously shown to be downregulated in CRC (Caldwell 2006), which is agreement with our speculation that one-carbon vitamin depletions upregulated *Wnt* signaling and thereby contribute to intestinal tumorigenesis. Another component which was experimentally validated is β-catenin, the accumulation and stabilization of which is critical in canonical *Wnt* signaling activation (Klaus 2008). *Axin2*, a downstream indicator of *Wnt* signaling, was marginally elevated (p = 0.054) in the one-carbon vitamin deficient group. The expression change of the remaining 3 genes were not experimentally validated. However, it is noteworthy that the expression changes of these genes by those B-vitamin depletion exhibit a pattern indicating the activation of *Wnt* signaling; the inhibition of *Sfrp1*, the *Wnt* antagonist, the elevation of β-catenin, a *Wnt* signaling transduction key component, and the mildly increased expression of *Axin2*, a downstream indicator of *Wnt* signaling.

CHAPTER 6

CONCLUSIONS

Overall, this study provides novel insight into the mechanisms of dietary modulation of colorectal cancer with a focus on one-carbon metabolism related vitamins driving alterations of miRNA expression which thereby regulate the tumorigenic *Wnt* pathway. By implementing a combinatorial approach to quantify *in vivo* differential expression of miRNAs through both microarray and Real-time PCR, and *in silico* prediction of miRNA targets, we demonstrated that the combined inadequacies of one-carbon vitamins including folate, riboflavin, vitamin B6 and B12 is sufficient to induce differential expression of miRNAs, which in consequence post-transcriptionally regulate genes within the *Wnt* signaling pathway.

This study presents a miRNA expression profile showing 18 miRNAs found to be either significantly ($p < 0.05$) or mildly ($p < 0.10$) differentially expressed in the colonic epithelium of mice fed the depleted diet when compared to the counterpart. *In silico* prediction of the targets of those 18 miRNAs identified 40 genes within the *Wnt* pathway to have homology with miRNA seed sequences within their 3'-UTR or protein coding sequence. Of the 6 genes tested for experimentally target validation, the expression of *Sfrp1* was shown to be significantly inhibited ($p < 0.05$) whereas β-catenin was shown to be significantly elevated ($p < 0.05$) with alterations of others in a fashion indicating the activation of *Wnt* signaling. These findings indicate that miRNAs may constitute a mechanism by which one-carbon B-vitamin depletions regulate the *Wnt* signaling pathway and thereby induce intestinal tumorigenesis. In summary, this study sheds light on the molecular mechanisms by which inadequate intake of folate and other metabolically related B vitamins modulate colorectal cancer risk, and provides novel insights into the practice

of colorectal cancer prevention by dietary management of one-carbon metabolism related vitamins.

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